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THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

110 EAST 59TH STREET  
NEW YORK, N. Y. 10022

Application For Research Grant

SEP 24 1974

Date: 9/5/74

1. Name of Investigator(s): (include Title and Degrees) J. Andrew Mitchell  
Assistant Professor  
Ph.D.
2. Institution & Address: Wayne State University  
School of Medicine  
Department of Anatomy  
540 East Canfield Street  
Detroit, Michigan 48201

3. Short Title of Project:

A Study of the Effects of Nicotine on Gestation in the Rat with Particular Reference to Implantation and the Time of Onset of Parturition

4. Proposed Starting Date: January 1, 1975

5. Anticipated Duration of this Specific Study: 2 years

6. Brief Description of Objectives or Specific Aims:

Considerable evidence indicates that nicotine adversely affects pregnancy in the rat. Among such disruptive effects are increased frequency of fetal resorption, reduced litter size and weight, and delayed parturition. The primary objective of the experiments embodied in this proposal is to gain a greater understanding of the means by which nicotine exerts disruptive effects on the normal course of pregnancy, and more particularly, how the chief alkaloid of tobacco reduces litter size and delays parturition. To this end, a variety of parameters will be measured to assess reproductive efficiency, time of implantation, fetal growth and development, and normalcy of parturition in nicotine treated vs. control rats. Several experimentally testable hypothesis are proposed. Specifically, experiments will be conducted to determine if nicotine induced reduction in litter size and delay in parturition are related to alterations in uterine sensitivity to implantation. The time of onset, magnitude and duration of sensitivity to decidualization will be compared in treated and control rats. In addition, the effects of nicotine on duration of gestation will be determined by an accurate measurement of the time of parturition.

7. Give a Brief Statement of your Working Hypothesis: please see page 4 of attached proposal.

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## 8. Details of Experimental Design and Procedures: (Attach Separate Pages)

Please see page 7 of attached proposal.

## 9. Physical Facilities Available (Where Other than Administering Organization Indicate Geographical Location)

A well equipped endocrinology laboratory and two animal rooms are available. In addition, complete histology and veterinary services are available as well as computer and photographic facilities.

## 10. Additional Requirements: None

## 11. Biographical sketches of all principal and professional personnel (append)

appended

## 12. List of publications: (Five most recent as pertinent) (append)

appended

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## 13. Budget (1st year)

## A. Salaries (Personnel by names)

## Professional

J. A. Mitchell Ph.D.

% time

Amount

35

REDACTED

## Technical

## Graduate Students:

1) D. R. Garris

2) to be determined

80

80

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Sub-Total

R

2000.00

1000.00

200.00

500.00

600.00

Sub-Total

## B. Consumable Supplies (list by categories)

Rat food &amp; litter, animal care fee

General reagents

Glassware, general lab supplies

Animals

## C. Other Expenses (itemize)

Publication cost

Page cost

Photography

Reprints

Computer Fee

2300.00

4300.00

150.00

60.00

150.00

250.00

Sub-Total

## D. Permanent Equipment (itemize)

Kymograph (research quality)

Surgical instruments

Roller-Smith Micro Balance

Animal cages

610.00

4910.00

360.00

200.00

380.00

300.00

## E. Overhead (15% of A+B+C)

1240.00

6150.00

Total 7387.50

6886.50

## Estimated Future Requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Overhead	Total
Year 2	R	1500.00	800.00	0	645.00	4945.00
Year 3	-	-	-	-	-	-

It is understood that the applicant and institutional officers in applying for a grant have read and found acceptable the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Signature

J. A. Mitchell, Ph.D.

Director of Project

577-1061

Telephone

Signature

Dr. T. J. Curtin

Business Officer of the Institution

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4.

## Other Sources of Financial Support

List financial support for research from all sources, including own institution, for this and/or related research projects.

## Current

Title of Project	Source	Amount	Duration
A Study of Intrauterine Oxygen Tension in the Guinea Pig with Particular Reference to the Availability of Oxygen at the Time of Implantation	Wayne State University General Research Support Grant	\$3,651.00	1973-1975

## Pending

Department of Anatomy will provide additional funding up to 20% of the total grant received

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## CURRICULUM VITAE

J. Andrew Mitchell

Date of Birth: REDACTEDPlace of Birth: Dallas, TexasMarital Status: REDACTEDEducation:

B.S.	1963	Southern Methodist University, Dallas, Texas Dr. John L. McCarthy, Research Advisor <u>Major:</u> biology, <u>Minor:</u> chemistry, philosophy
Ph.D.	1969	University of Kansas, Lawrence, Kansas Dr. Jerome M. Yochim, Dissertation Advisor <u>Major:</u> physiology, <u>Minor:</u> biochemistry

Positions:

1962-1964	Teaching Assistant in Physiology, Biology and Microbiology
1966-1968	Teaching Assistant in Physiology, Biochemistry and Advanced Endocrinology
1969-1972	Post-Doctoral under Dr. Vaughn Critchlow, Baylor College of Medicine, Department of Anatomy, Houston, Texas
1972-	Assistant Professor of Anatomy, Department of Anatomy, Wayne State University School of Medicine

Awards & Fellowships:

1966-1967	University of Kansas Research Assistantship
1967-1968	University of Kansas Research Assistantship
1967	National Science Foundation Summer Graduate Traineeship
1968	William King Candlin Award in Physiology
1969-1971	Public Health Service Postdoctoral Research Fellowship

Memberships:

REDACTED

Foreign Languages:

Reading knowledge of French and German

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Research Interests:

Physiological and biochemical aspects of nidation  
Photoperiod effects on reproductive phenomena  
Regulation of ACTH and growth hormone secretion  
Neural regulation of circadian ACTH release  
Neural regulation of gonadotrophin secretion

Publications:

- McCarthy, J.L., A.L. Brodsky, J.A. Mitchell and R.F. Herrscher 1964  
Thin-Layer Chromatography of Adrenal Corticoids: Observations on  
Changes in Mobilities, Analytical Biochemistry 8:164-170.
- Mitchell, J.A. and J.M. Yochim 1968 Relation of HCG-Induced Ovulation  
to the Production of Prolonged Diestrus in the Adult Rat,  
Endocrinology, 82:1142-1148.
- Mitchell, J.A. and J.M. Yochim 1968 Measurement of Intrauterine  
Oxygen Tension in the Rat and Its Regulation by Ovarian Steroid  
Hormones, Endocrinology, 83:691-700.
- Mitchell, J.A. and J.M. Yochim 1968 Intrauterine Oxygen Tension During  
the Estrous Cycle in the Rat: Its Relation to Uterine Respiration  
and Vascular Activity, Endocrinology, 83:701-705.
- Yochim, J.M. and J.A. Mitchell 1968 Intrauterine Oxygen Tension in  
the Rat During Progestation: Its Possible Relation to Carbohydrate  
Metabolism and the Regulation of Nidation, Endocrinology, 83:706-713.
- Yochim, J.M. and J.A. Mitchell 1970 Influence of Environmental  
Lighting on Decidualization in the Rat, Endocrinology, 87:465-471.
- Mitchell, J.A. and J.M. Yochim 1970 Influence of Environmental  
Lighting on Duration of Pregnancy in the Rat, Endocrinology,  
1967 87:472-480. National Science Foundation Grant #10671-67  
1968 William King Gendlin Award in Physiology
- Mitchell, J.A., M. Hutchins, W. Schindler and V. Critchlow 1972  
Plasma Growth Hormone Levels in Rats with Increased Naso-Anal  
Lengths Due to Hypothalamic Surgery, Neuroendocrinology, 10:31-45.
- Mitchell, J.A., M. Hutchins, W.J. Schindler and V. Critchlow 1973  
Increases in Plasma Growth Hormone Concentration and Naso-Anal  
Length in Rats Following Surgical Isolation of the Medial Basal  
Hypothalamus, Neuroendocrinology, 12:161-173.
- Mitchell, J.A., Ependymal Tanycytes and Anterior Pituitary Function  
(a review submitted for publication)
- Belsare, D.K., J.A. Mitchell and V. Critchlow 1974 Neural Structures  
that Trigger Ovulation in the Rat, Journal of Reproduction  
and Fertility, 38: 242-243.

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**Abstracts: Biological and biochemical aspects of nidation**

Photoperiod effects on reproductive phenomena

Mitchell, J.A. and J.M. Yochim: 1969 Influence of Photoperiod on the Duration of Gestation and the Time of Parturition in the Rat, The Anatomical Record, 163: 230.

Mitchell, J.A. and J.M. Yochim: 1969 The Influence of Photoperiod on the Onset, Duration and Magnitude of Uterine Sensitivity to Decidualization in the Rat, The Anatomical Record, 163: 317.

Thin-Layer Chromatography of Adrenal Corticoids: Observations on

Mitchell, J.A., D.K. Belsare, M. Hutchins, W.J. Schindler and V. Critchlow 1971 Increases in Growth and Plasma Growth Hormone Levels

Following Surgical Isolation of Medial Basal Hypothalamus in Female Rats, The Endocrine Society, Program of the 53rd Meeting, P.A-89, Paper No. 179.

Mitchell, J.A., D. Belsare, M. Hutchins, W. Schindler and V. Critchlow 1972 Increases in Growth and Plasma Growth Hormone Levels Following Horizontal Cuts in the Ventromedial Hypothalamus in Female Rats. The Anatomical Record, 172: 368.

Mitchell, J.A. and J.M. Yochim: 1969 Anesthetizing by the Vaginal Route the Effect of Light on the Duration of Gestation

Yochim, J.M. and J.A. Mitchell: 1970 Influence of Environmental Lighting on Reproduction in the Rat, Endocrinology, 87: 472-480.

Lighting on Duration of Pregnancy in the Rat, Endocrinology, 87: 472-480.

Mitchell, J.A., D.K. Belsare, M. Hutchins, W.J. Schindler and V. Critchlow: 1971

Increases in Plasma Growth Hormone Levels Following Horizontal Cuts in the Ventromedial Hypothalamus in Female Rats

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Abstracts:

Invited Lecturer:

Matchett, J.A. and J.N. Yocum. 1969. Influence of Photoperiod on the C.S. Mott Center Colloquium "Endocrine Functions of the Hypothalamus." Lecture Title: "Structure-Function Relationships in the Hypothalamus" February 7, 1974.

Matchett, J.A. and J.N. Yocum. 1969. The Influence of Photoperiod on William Beaumont Hospital Neurology Residency Program "Basic Concepts in Neuroendocrinology" Neurological Abstracts, 1974: 31. March 1, 1974.

Matchett, J.A., D.W. Beltsare, M. Hutchins, W.L. Schindler and V. Orshchlow. 1977. "Influence of Growth and Plasma Growth Hormone Levels Following Surgical Isolation of Medial Basal Hypothalamus in Rats."

Invited Panel Discussant:

Society for Neuroscience - Michigan Chapter. 1974. "Neuroanatomical Substrates of Neuroendocrine Intergration"

Meetings Attended:

D. Beltsare, M. Hutchins, W. Schindler and V. Orshchlow

7th Annual Scanning Electron Microscope Symposium  
Chicago, Illinois, April 8 - 11, 1974.

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CURRICULUM VITAEName: Lecturer:

David R. Garriss

Address:

Mott Center Colloquia Department of Anatomy  
 Wayne State University  
 School of Medicine  
 G. H. Scott Hall of Basic Medical Sciences  
 540 E. Canfield  
 William Beaumont Hospital Residency Program  
 Detroit, Michigan 48201  
 "Basic Concepts in Neuroendocrinology"  
 March 1, 1974.

Birth Date:

REDACTED

Marital Status:

REDACTED

Education:

Society for Neuroscience - Michigan Chapter  
 Anatomical Substrate of Neuroendocrine Regulation

University	Major	Minor	Degree
Eastern Michigan University	Biology	Chemistry	B.S.
Ypsilanti, Michigan			
1969-1973			
Wayne State University	Anatomy	Physiology	
Detroit, Michigan		Biochemistry	
1973-1974			

Memberships:

REDACTED

Awards and Fellowships:

National Science Foundation COSIP Student Eastern Michigan University  
 Department of Chemistry 1973  
 National Science Foundation COSIP Student Eastern Michigan University  
 Department of Biology 1973  
 Wayne State University Graduate Teaching Assistantship 1973-1974

Teaching Experience:

Teaching Assistant (EMU) Histology  
 Teaching Assistant (WSU) Anatomy, Neuroanatomy

Meetings Attended:

The Society for the Study of Reproduction Ottawa, Canada August, 1974

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Name:

David R. Garwin

Address:

Department of Anatomy  
Wayne State University  
School of Medicine  
5. E. Scott Hall of Basic Medical Sciences  
540 E. Canfield  
Detroit, Michigan 48201

Birth Date:

October 28, 1951

Master's A Study of the Effects of Nicotine on Gestation in the Rat

Education with Particular Reference to Implantation and the Time of

Onset of Parturition. Biology

Chemistry

B.S.

Wayne State University, Detroit, Michigan 1974-1977

Wayne State University, Detroit, Michigan

Physiology,  
Endocrinology

Supervisor: J. A. Mitchell

Advisor and Sponsor: Wayne State University

National Science Foundation School of Medicine  
Department of Anatomy

Eastern Michigan University

National Science Foundation COSIF Student  
Department of Biology

Eastern Michigan University

Wayne State University, Detroit, Michigan

Teaching Assistant (HIST)

HISTOLOGY

Teaching Assistant

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### 1. Present State of Knowledge

The results of numerous studies have established that nicotine may interfere with the normal course of pregnancy in the rat. It has been claimed that the administration of nicotine to pregnant rats results not only in reduced litter size (20-22, 38, 40, 42) and litter weight (2-5, 15, 18, 19, 25, 26) but also in increased frequency of stillbirths (4, 11, 22) with particular reference to implantation and the time of and infant mortality (2-4, 10, 11, 14, 20, 32, 34).

Onset of Parturition

Among the most systematic and informative studies of the actions of nicotine on pregnancy are those of Becker et al., (2-5). When twice daily sc injections of 3.0 or 5.0 mg/kg of nicotine were administered throughout pregnancy, the onset of parturition was delayed as much as 3 to 5 days, the mean delay resulting from the higher dose being 2 days (4).

Also, the young weighed less and were retarded in development relative to controls.

In another study, Becker et al., (5) again employed the 5 mg/kg dose to prolong gestation. However, for comparison non-nicotine treated rats were given chorionic gonadotrophin in order to prolong pregnancy. Although equivalent delays in parturition were achieved in the two groups (all were delivered by Ceasarean section of Day 21, 22, 23, or 24), the characteristic of the young were markedly different. Young born following hormonally prolonged gestation evidenced the typical post-maturity syndrome, i.e., greater body weight and development compared with

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~~neonates born at term~~ In contrast the neonates of nicotine treated rats, though subjected to an equivalent delay in parturition, were markedly lighter in weight and retarded in development. has been claimed that the administration of nicotine. More recently, Hudson and Timiras (17) investigated the effects of nicotine on reproductive capacity and length of gestation, as well as on litter size and viability. Injection of 5 mg/kg of nicotine, administered sc twice daily throughout pregnancy, prolonged gestation by 2-3 days; the most protracted pregnancy was of 27 days duration. However, in contrast with the results of Becker et al., (4, 5) birth weights were not statistically different between treated and control rats. Furthermore, no mention was made of retarded development.

Birth weights of nicotine treated rats did not differ from those of control rats in spite of prolonged gestation, which suggests that either the rate of fetal growth was reduced throughout gestation, or that the rate of growth was normal but slow in starting. The failure of prolonged gestation in nicotine treated rats to result in larger and more mature neonates favors the latter interpretation and suggests that nicotine delays implantation. A delay in uterine sensitivity would produce a delay in implantation which in turn could be reflected as a delay in parturition. Such a delay in nidation would be consistent with the birth of young of normal weight and development as reported by Hudson and Timiras (17).

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neonates. However, other explanations for the observed delay in parturition are possible. The prolongation in gestation reported by Becker et al. (4, 5) was associated with reduced fetal bulk. A reduction in fetal mass may contribute toward a delay in parturition (29). It is also possible that the nicotine reduces fetal mass by reducing the magnitude of uterine sensitivity to the blastocyst, thereby diminishing litter size. Reduction in litter size following administration of nicotine has been observed (19, 33, 35). Another possibility is that nicotine may delay the onset of parturition, not by delaying implantation but by retarding the rate of fetal growth by suppressing maternal food intake (4). The proposed experiments are designed to discriminate between these various possibilities.

## 2. Specific Objectives

The primary objective of the experiments embodied in this proposal is to gain a greater understanding of the means by which nicotine exerts disruptive effects on pregnancy in the rat, and more particularly, how the alkaloid brings about reduction in litter size and delay in parturition.

The following experimentally testable hypotheses are proposed:

- I. That nicotine reduces uterine sensitivity to implantation of the blastocysts.
- II. That nicotine delays the normal time of onset of uterine sensitivity to implantation of the blastocysts.

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III. That the nicotine-induced delay in time of parturition in rats is a reflection of the delay in the time of implantation of the blastocyst. was associated with reduced fetal weight. The following is a brief resume of why it is reasonable to assume that nicotine may alter endometrial physiology:

Ample evidence has been marshalled to support the long held contention that uterine sensitivity to decidualization is a reliable model for studying uterine receptivity to the rat blastocyst (13). Because induction of the decidual cell reaction has proved so useful in investigating the process of nidation itself, considerable work has been directed toward determining the physiological and metabolic conditions which are necessary to support the response.

Not only has the temporal course of uterine sensitivity to decidualization been worked out in detail (38), but the hormonal balance requisite for optimal deciduoma formation has been established (37). It is now known that the magnitude of the decidual response as well as the timing of sensitivity is regulated by an estrogen-progesterone interplay. Although a decidual response may be evoked in the presence of progesterone alone, it is submaximal in magnitude; estrogen must also be present for maximal responsiveness to occur. Although estrogen potentiates the uterine sensitizing effect of progesterone, it also curtails the period during which a maximal decidual reaction can be evoked (38).

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Therefore, whereas progesterone renders the endometrium receptive to the blastocyst, estrogen regulates the magnitude and duration of receptivity. The regulation of the secretion of both of these ovarian steroid hormones is, of course, under the control of the anterior pituitary and thereby ultimately regulated by the hypothalamus. The process of nidation is dependent on the proper synchronization of ovulation and sensitivity of the endometrium to the presence of the blastocysts (26). Furthermore, like decidualization, the development and maintenance of uterine sensitivity to implantation is dependent upon the proper balance of ovarian steroids (13), the secretion of which is, at least partially, regulated by lutenizing hormone (LH) and prolactin from the anterior pituitary (1). Recently, studies by Blake, et al. (6-8) have established that nicotine may alter the timing of the ovulatory surge of LH from the anterior pituitary via action on the hypothalamus. Although the study was confined to the effects of nicotine on the timing of the critical period for LH release in ovulation, the findings raise the possibility that nicotine may similarly alter LH release not only prior to ovulation, but perhaps also during early pregnancy. Similarly, nicotine administration inhibits the proestrous surge of prolactin (9). Thus, it is possible that appropriately timed administration of nicotine may alter LH and/or prolactin release in such a

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Therefore, whereas progestogens render the endometrium in a manner as to impair the delicate balance of ovarian steroids required to maintain uterine receptivity to the blastocyst, and thereby delay and/or impair uterine sensitivity to implantation.

under the control of the anterior pituitary and thereby. In addition to the possibility that nicotine may alter the course of nidation via its action on the CNS it may also influence implantation via local uterine effects.

For example, it has been suggested that the site of blastocyst invasion is influenced by the abundance of intrauterine vasculature, implantation occurring preferentially near an area of increased intrauterine  $pO_2$  (39). Since nicotine

is known to be a vaso-active substance, it is possible that the alkaloid may influence blastocyst attachment by altering the endometrial vasculature, thereby modifying oxygen availability.

That nicotine may exert an influence in implantation via action of the blastocyst itself as well as on the endometrium is suggested by the study of Fabro and Sieber (16). Following iv injection of G-( $^3H$ )-nicotine into rabbits, radioactivity was detected in the endometrium, uterine fluid, and blastocyst. The data suggest that nicotine may be taken up prior to implantation by the blastocyst.

### 3. Experimental Designs

#### A. Treatments

Hypotheses I & II, that nicotine reduces uterine sensitivity to implantation and/or delays the onset of such sensitivity will be tested in the following ways:

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1) Comparing the time of onset, duration and magnitude of uterine sensitivity to decidualization in control vs. nicotine treated rats.

2) Estimating of the time and frequency of implantation

in control vs. nicotine treated rats by, a) direct visual detection of early implant sites, and

b) determination of the time during which free

blastocysts can be recovered from the uterus by flushing.

Hypotheses III, that the delay in parturition observed in nicotine treated rats reflects the delay in the time of implantation of the blastocyst, will be tested by accurately measuring the time of parturition in control vs. nicotine treated rats.

The morning on which spermatozoa are found in the vaginal lavage will be designated Day 0 of pregnancy. Insemination will arbitrarily be assumed to have occurred the previous midnight. For each experiment, inseminated rats will be assigned randomly to one of two groups: nicotine treated and control. Rats in the treated group will be assigned randomly to one of 3 dosages of nicotine: 2, 6 and 10 mg/kg of nicotine per day. The alkaloid will be injected sc twice daily in 2 equal doses, the first administered between 8:00-9:00 am, the second between 5:00-6:00 pm. The 2 mg/kg dose, while pharmacological in the rat, is comparable to that received by a heavy smoker according to the studies of Wolff *et al.*, (36).

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In addition to studying the effects of different dosages of nicotine on the parameters selected, the effects of duration of treatment will be studied. In one series of experiments, the various dosages of nicotine will be administered only during the initial 8 days of pregnancy or pseudopregnancy. In another series, the nicotine treatment will be continued throughout the duration of pregnancy. In addition, other dose levels and schedules may be used, as suggested by the experimental results obtained.

#### B. Reagents

The nicotine to be used will be of the purest grade (Eastman Kodak) and prepared as a 2% stock solution in 0.85% NaCl solution for dilution as necessary to prepare the proper dosage. The stock solution will be prepared at 2-3 day intervals, stored in amber glass bottles and refrigerated.

Control rats will receive equivalent volumes of 0.85% NaCl solution via sc injections administered on the same schedule as the nicotine treated rats. Otherwise, treated and control animals will be cared for in identical fashion.

### 4. Materials and Methods

#### A. Animals, Environment and Routine Procedures

Mature virgin female rats of the Holtzman strain, 200-300 g body weight, will be maintained under controlled environmental conditions with respect to room temperature

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(20 - 25°C), humidity (40-60%) and photoperiod (14 hr. light, 10 hr. darkness). Illumination will be provided by standard General Electric "cool white" fluorescent lights (color temperature 4000 - 5000 K) regulated by an automatic timer. The intensity of illumination at the levels of the cages will range from 430 to 1180 lumens/m<sup>2</sup>. Animals will be housed 1/cage and provided free access to Purina Lab Chow and water. Vaginal smears will be recorded daily for at least 3 complete estrous cycles prior to breeding or induction of pseudopregnancy.

Pseudopregnancy will be induced by mechanical stimulation of the uterine cervix with a glass rod during proestrus and estrus. Day 0 of pseudopregnancy will be defined as the last day of vaginal cornification prior to the onset of the luteal phase; Day 1 will be the day on which the vaginal smear is predominately leukocytic.

#### B. Measurement of Time of Onset of Parturition

The time of onset of parturition will be measured as previously described (24). On the 19th day of pregnancy, each rat will be transferred to a special cage arrangement which permits the determination of the time of onset of parturition with no disturbance to the animal (Fig. 1). A standard wire-bottom rat cage (7" wide, 9" long & 7" high) will be provided with an aluminum floor which slopes to a 1" x 2" opening at the rear of the cage. Below the opening, chart paper will be moved at a known rate of speed by a

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horizontally arranged kymograph. During parturition, light, amniotic fluid and blood will flow through the hole to drop below and stain the moving chart paper. By measuring the position of the initial stain, the time of onset of parturition can be calculated within  $\pm 15$  minutes. Direct observations have confirmed that this procedure provides a good index of the time of onset of delivery.

#### Treatment of Data:

Though the apparatus for recording delivery will allow determination of parturition to within  $\pm 15$  minutes, mean times of delivery will be calculated for 6 hour intervals: all deliveries within  $\pm 3$  hours of the plotted time will be pooled to produce the plotted value. The results will be calculated as the percent of the total experimental population which delivered every 6 hours during Days 21-24 (or whatever span of time is appropriate for the experimental results) of gestation. Differences of less than 20% between comparable experiments will be arbitrarily considered to be of borderline biological significance. To determine the statistical significance of the data, the mean time of delivery will be calculated for each experiment, and analysis of variance will be performed following a confirmation of homogeneity of variance.

#### C. Induction of Decidualization

Deciduomata will be induced by surgical traumatization as previously described (40). In brief, each rat will be

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horizontally arranged kymograph. During parturition, laparotomized by a mid-line incision and one or both uterine amniotic fluid and blood will flow through the sole to horn horns will be traumatized by inserting a knife-tipped needle below and near the moving chart paper. The pressure will be measured into the uterine lumen by way of a small incision placed near the junction of the horn and the cervix. The blade will be pushed into the lumen and completely to the ovarian end of the uterus. The knife edge will be pressed against the entire length of the antimesometrial surface of the uterus as it is withdrawn.

Decidualization will be induced in each rat during one of 6 times: Day 2 midnight; Day 3 noon or midnight; Day 4 noon or midnight; Day 5 noon. Five days following uterine traumatization, the rats will be weighed and sacrificed by ether asphyxiation; the uterine cornua will be removed, placed on moist bibulous paper, and trimmed of adhering fat and mesentery. The cornua will be separated and weighed on a torsion balance to the nearest 0.5 mg.

#### Treatment of Data:

Decidual sensitivity will be expressed in 2 ways: as mg/cornu, and as percent of an arbitrary average response to trauma during the period of maximal sensitivity in normal rats (38). Under these conditions, 100% sensitivity = 2000 mg/cornu. To simplify the interpretation of results, the "onset" of decidual sensitivity will be defined as responses between 40 and 80% of maximal. Statistical analysis of the data will be performed using the one-way analysis of variance.

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### 5. Additional Experiments and Observations

1) Changes in timing and frequency of implantation

A. Recovery of Blastocysts. A small incision is made near the uterine horn. As an additional test of the ability of nicotine to alter the time of implantation, the rate of recovery of blastocysts from the uterus during the preimplantation period by saline flushings will be determined. Normally, under the conditions of the present experiments, by early Day 5 nidation has proceeded to a point that blastocysts can no longer be flushed from the uterus. Therefore, a delay in implantation will be suggested if unattached blastocysts are obtainable from nicotine treated rats at significantly later times than in control rats.

An another indication of whether or not nicotine administration results in delayed nidation, the mesometrial-anti-mesoentrial width of the implantation sites will be measured on Day 8 of treated and control rats. Such measurements will provide an approximate indication of when implantation occurred (30).

#### B. Early visual Detection of Implantation

The benzyl-benzoate clearing technique described by Orsini (27) will be used to render implantation sites visible. After fixing, dehydrating, bleaching and clearing, rat uteri illuminated by oblique light reveal nidation sites as early as 5 days and 9 hours following insemination (28). A delay

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in implantation will be indicated if nidation sites are not visible in nicotine treated rats until significantly later times than in control rats.

2) Changes in Birth Weight and Growth, Placenta Weight, the Frequency of Resorption, and Maternal Food Intake.

A. Birth Weight

To determine whether or not reduction of fetal mass occurs, and if so, by what mechanism(s) it takes place, a variety of experiments and observations will be performed. Following delivery, each pup will be weighed, sexed, measured for length and examined for the degree of development and presence of congenital defects. The total litter and mean neonate weights will be computed and percentage of live births noted.

B. Development

Neonates will be relaxed with ether anesthesia and the naso-anal length measured to the nearest mm with vernier calipers. In addition, the stages of development of each newborn will be assessed using the method of Christie (12).

This method requires the careful examination of each newborn for specific morphological characteristics, the presence or absence of which permits an estimation of the stage of development with an accuracy of  $\pm 1/2$  day.

C. Growth

As an additional indicator of growth, rate of fetal bone growth will be assessed using procion dyes. The procion dyes

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to be used are derivatives of mono-chlorotriazinyl, are water soluble, and react irreversibly with osseous tissue. Since such dyes do not dissolve during the histological preparation of stained bone, they act as indicators of bone growth (26, 31).

Although the details of timing, dosage, etc., remain to be determined, in general, the method will consist of an initial iv injection of Procion Brilliant Red II-8BS, followed at an appropriate interval by an injection of Procion Brilliant Purple H-3Bs. Shortly after birth, and following measurement of other parameters, the femurs of each neonate will be dissected free, cleaned, weighed, measured for length, and fixed for histological examination. Following decalcification and sectioning, the distance between the red and purple dye lines (resulting from the administration of Procion Brilliant Red and Purple, respectively), will be measured with an ocular micrometer. The rate of femur growth during the interval between dye injections will be computed. Three measurements will be made on each femur and the mean distance between dye lines determined for each. In an effort to eliminate biased measurements, the identity of each specimen will be coded so as to prevent the observer from knowing the treatment administered. All indices of growth, ie., naso-anal length, body weight, femur length and weight, as well as rate of femur growth, will be subjected to appropriate statistical analysis to determine if there are differences between the experimental and control groups.

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D. Placental Growth - types of mono-chlorotriaziny), are water soluble. The effects of nicotine on placental growth will be determined and morphologic evidence of altered placental function will be sought. In randomly selected animals, (4, 34), direct visual observations will be made to detect the onset of labor. Following delivery of the first pup, the mother will be anesthetized and the remainder of the litter delivered by Caesarean section in order to obtain intact placentae. Such placentae will be cleaned, blotted dry, and weighed. Placental diameter will be measured with vernier calipers, prior to fixation for histological examination.

E. Frequency of Resorption

To determine if nicotine increases the frequency of fetal resorption, each mother rat will be sacrificed by ether anesthesia and the uteri removed, cleaned and weighed. The number and distribution of implantation sites will be determined. The frequency of resorption will be computed for each litter by comparing the number of young in the litter vs. the number of implantation sites.

F. Changes in Maternal Food Intake.

In order to determine if nicotine administration results in reduced maternal food intake, food will be provided in spill proof containers and daily food and water consumption will be measured. Should nicotine treatment be associated with a significant reduction in food consumption, paired feeding

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D. Placental Growth

experiments will be initiated to determine what effect, the effects of nicotine on placental growth will be in any, and equivalent reduction of food intake exerts on fetal growth and the time of parturition in control rats. Controls will be paired randomly with nicotine treated rats inseminated on the same evening. Food intake of nicotine treated animals will be measured daily, and the paired control rat provided with only an equivalent quantity of food. Paired animals will be observed for time of parturition; litters and post-parturient mothers will be subjected to the standard series of measurements to determine if the effects of nicotine are possibly brought about by reduced caloric intake.

other animals of the litter removed, checked and verified. The frequency of resorption will be computed for

In order to determine if nicotine administration results

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experiments will be initiated to determine what effect, in any and equivalent reduction of food intake exerts on fetal growth and the time of parturition in control rats. Controls will be paired randomly with nicotine treated rats. Food intake of nicotine treated animals will be recorded and the same amount of food will be provided to the control rats. Food intake of nicotine treated animals will be recorded for time of parturition.

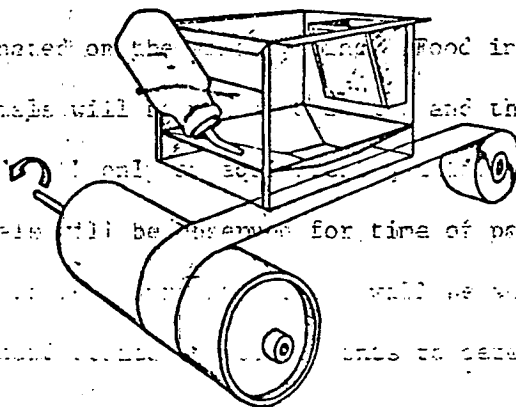


FIG. 1. Apparatus used to determine time of onset of parturition in the rat.

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